Probing Interactions between Aggrecan and Mica Surface by the Atomic Force Microscopy

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ABSTRACT: Aggrecan is a bottlebrush shaped macromolecule found in the extracellular matrix of cartilage. The negatively charged glycosaminoglycan (GAG) chains attached to its protein backbone give aggrecan molecules a high charge density, which is essential for exerting high osmotic swelling pressure and resisting compression under external load. In solution, aggrecan assemblies are insensitive to the presence of calcium ions, and show distinct osmotic pressure versus concentration regimes. The aim of this study is to investigate the effect of ionic environment on the structure of aggrecan molecules adsorbed onto well-controlled mica surfaces. The conformation of the aggrecan was visualized using Atomic Force Microscopy. On positively charged APS mica the GAG chains of the aggrecan molecules are distinguishable, and their average dimensions are practically unaffected by the presence of salt ions. With increasing aggrecan concentration they form clusters, and at higher concentrations they form a continuous monolayer of conforming molecules. On negatively charged mica, the extent of aggrecan adsorption varies with salt composition. Understanding aggrecan adsorption onto a charged surface provides insight into its interactions with bone and implant surfaces in the biological milieu. © 2010 Wiley Periodicals, Inc.[†] J Polym Sci Part B: Polym Phys 48: 2575–2581, 2010

KEYWORDS: adsorption; aggrecan; atomic force microscopy; osmotic pressure

INTRODUCTION Aggrecan is a bottlebrush shaped macromolecule found in the extra-cellular matrix of cartilage.[1–5] It consists of a linear protein core (220–250 kDa) with three globular domains (GD1, 2, and 3) along it (Fig. 1).[1] Between GD2 and GD3, a number of negatively charged chains of glycosaminoglycans (GAGs) are attached to the protein core.[4,6,7] The Keratan Sulfate GAG chains are shorter (∼15–20 nm), numbering up to 30–60 per aggrecan molecule, and have about 50 negative charges per chain.[1,6,8] The Chondroitin Sulfate GAG chains are longer (∼20–60 nm), their number is about 100 per aggrecan molecule, and have up to 100 negative charges per chain.[1,6–8] A two-dimensional representation of the aggrecan bottlebrush structure is shown in Figure 1, along with its typical dimensions. Aggrecan is usually polydisperse (1–5 $\times$ 10^6 Da), even when isolated from a single tissue source.[7,9] There are variations in the length of the protein core between GD2 and GD3 domains, and in the number of the GAG chains.[2,4,9] In cartilage aggrecan/hyaluronic acid assemblies are enmeshed in a network of collagen fibers.

Aggrecan assemblies enable cartilage to bear compressive loads and absorb mechanical shock.[8,10,11] Aggrecan is negatively charged and in solution it exerts a large osmotic swelling pressure. The osmotic swelling pressure resists compressive loads on the tissue, and keeps the tissue hydrated.[12,13] In the bottlebrush structure, the length of the GAG chains are within few multiples of their persistence length.[10] Due to the semirigid nature of the GAG chains and the electrostatic repulsion between them, the aggrecan bottlebrush itself has a large persistence length (∼80–110 nm).[7] Under physiological conditions aggrecan assumes an extended conformation, which is critical for maintaining a large water fraction for the diffusion of nutrients[8] and effective damping of dynamic loads.[10,14,15]

The osmotic pressure Π of aggrecan solutions exhibits different dependencies in different concentration regimes.[11] Below $c \approx 0.005$ g/cm^3, Π increases linearly with the aggrecan concentration. Linear dependence is typically observed in dilute polymer solutions, where the osmotic pressure is dominated by the kinetic mobility of the noninteracting particles. Between 0.005 and 0.015 g/cm^3, Π displays a weaker than linear dependence on aggrecan concentration, which can be attributed to self-assembly among the aggrecan bottlebrushes. Above 0.015 g/cm^3 Π follows a nearly quadratic dependence on the aggrecan concentration indicating that Π is dominated by binary monomer–monomer interactions.

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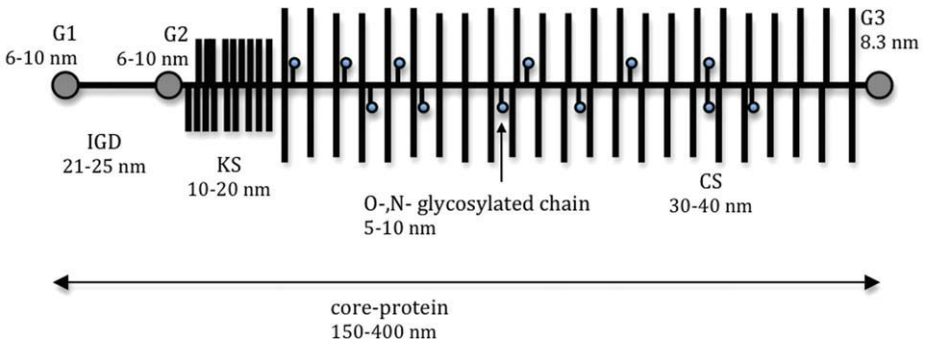

FIGURE 1 Two dimensional representation of the aggrecan molecule with typical dimensions reported in literature.[1–3] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Recent observations show that aggrecan assemblies in solution are insensitive to the presence of calcium ions.[11] Small-angle scattering measurements (SANS and SAXS) revealed that the structure of these systems is practically unaffected by changes in the ionic environment (ionic strength, counter-ion valence). The insensitivity to calcium ions is particularly important to the biological function of aggrecan.[16–19] Osmotic pressure measurements show that the addition of $CaCl_2$ reduces the osmotic pressure but does not influence the power-law behavior in each concentration regime. We found that (i) the concentration thresholds between these regimes are practically independent of the calcium content, and (ii) the shape of the Π versus c curves remains unchanged. Addition of 100 mM $CaCl_2$ reduces the value of Π by a factor of $\sim$2. The observed insensitivity of aggrecan assemblies to changes in the ionic environment is in sharp contrast to the behavior of typical linear synthetic and biological polyelectrolytes, such as polyacrylic acid or DNA, which precipitate in the presence of multivalent ions.[11,20–23]

The first atomic force microscopy (AFM) image of aggrecan was published by Ortiz and coworkers[7] on positively charged APTES-mica. They investigated the nanomechanical properties of aggrecan by combining microcontact printing and atomic force microscopy.[24–30] The lateral and compressive stiffness of end-grafted monolayers were estimated by probing the conformation and compressibility of aggrecan functionalized surfaces in aqueous salt solutions. Although these elegant measurements allow the determination of the interaction forces between the bottlebrushes, they do not yield direct information on the effect of salt on the adsorption and assembly of aggrecan molecules.

In this study, we investigate the structure of adsorbed aggrecan molecules on charged model substrates under different salt conditions. As in the physiological milieu aggrecan molecules are in contact with solid surfaces (e.g., bone, implants), a better understanding of the adsorption process may contribute to a more complete model of cartilage/skeletal metabolism. Adsorbed aggrecan molecules and their assemblies are imaged using AFM. As aggrecan is negatively charged, it is expected that electrostatic interactions govern its adsorption characteristics. We compare aggrecan adsorption on positively charged APS-treated mica and negatively charged untreated mica. We investigate the effect of ions on the morphology of adsorbed aggrecan molecules, and determine the average size (height-width dimensions) of their GAG regions.

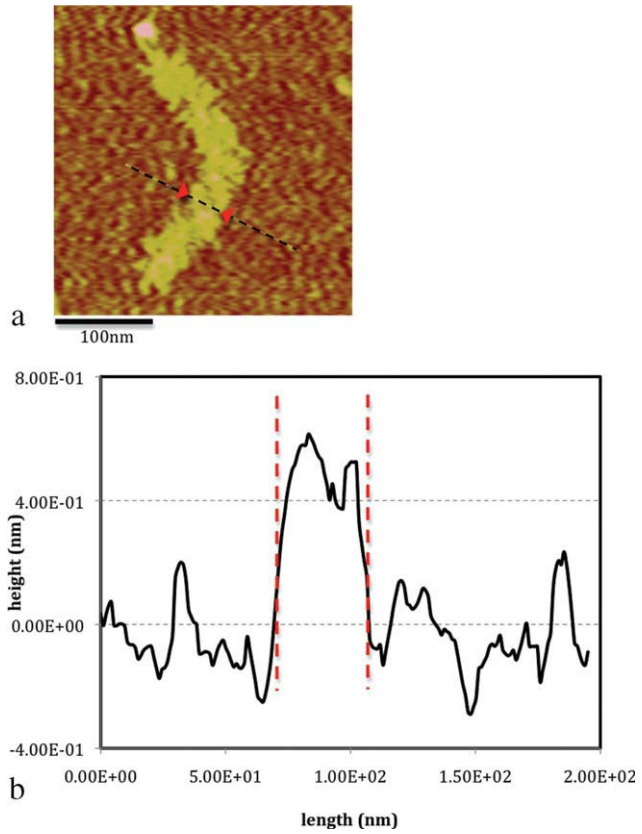

FIGURE 2 Measurement of height and width along a cross section of the GAG region of an aggrecan molecule (a) using the section analysis feature of the Nanoscope Imaging Software. The plot (b) shows the height profile in nm along a black line drawn across the GAG region of the aggrecan bottlebrush in (a). The width is determined from the horizontal distance between two vertical lines (dashed) which demarcate the cross section of the molecule and which corresponds to the arrows in (a). The maximum height along the selected cross section is reported for the height of the molecule. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

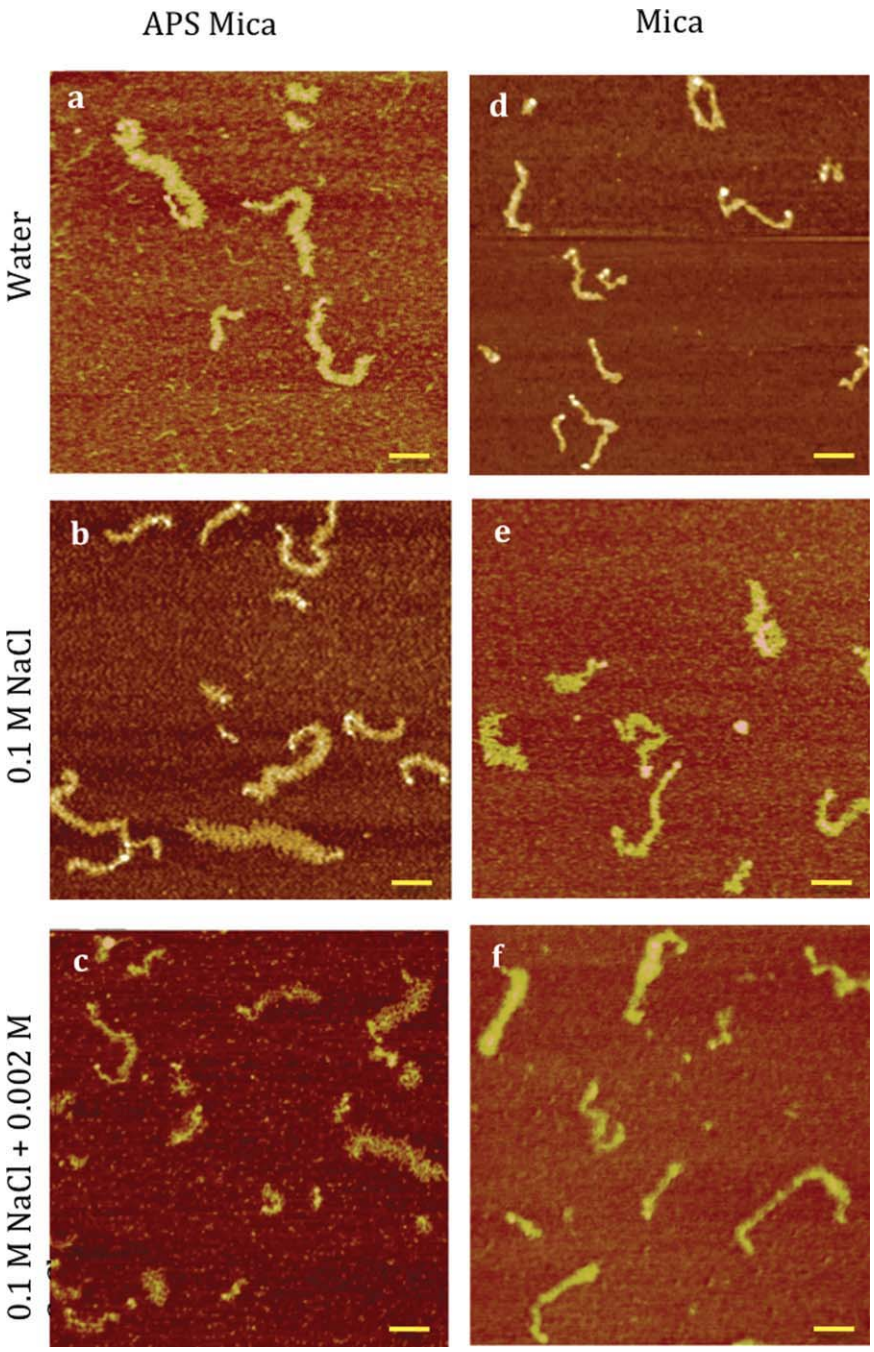

FIGURE 3 Aggrecan (1 μg/mL) adsorption on untreated mica and APS-mica surfaces for different salt conditions. GAG chains are visible on APS mica and for the 0.1 *M* NaCl condition on mica. The globular domains are visible as bright spots along the protein core. Scan area: 1 $\times$ 1 μm^2; scan frequency: 1.5 Hz, scale bar: 100 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We also identify changes in aggrecan adsorption patterns with increasing aggrecan concentration.

EXPERIMENTAL

Aggrecan from bovine articular cartilage was obtained form Sigma-Aldrich (St. Louis, MO). APS [1-(3-Aminopropyl) Silatrane] mica was prepared by coating freshly cleaved mica with 50 μL of 167 nM APS solution for 30 min, washing repeatedly, and drying in an Argon stream. The APS solution was prepared as described by Shlyakhtenko et al.[31] The nominal surface charge density of untreated mica is 2 negative charges/nm^2.[32] APS mica is known to be weakly positive charged, but to our knowledge, there is no report on its surface charge density.[31]

For the adsorption studies, a 7 μL drop of the aggrecan solution (c = 1 μg/mL, pH = 7) was placed on freshly cleaved

mica or APS-mica for 3 minutes, then washed with ultrapure, deionized water to remove salts, and dried in a gentle stream of argon. (In a separate experiment we found that 3 minutes adsorption time was sufficient to attain quasiequilibrium.) To investigate the effect of salt, we imaged aggrecan that was dissolved in (a) water, (b) 0.1 *M* NaCl, and (c) 0.1 *M* NaCl containing 0.002 *M* $CaCl_2$. (0.002 *M* is the typical calcium ion concentration found in cells and extracellular matrix.[33]) At the aggrecan concentrations used in this study, we estimated that a 0.002 *M* $CaCl_2$ solution has at least 10 calcium ions for each aggrecan negative charge. As aggrecan from the salt-free aqueous solution adsorbs poorly on untreated mica, for this case only, samples were prepared by placing 3 μL aggrecan solution on the surface and allowing it to air dry without washing.

AFM imaging was performed in air in the tapping mode. Silicon cantilevers were used (OMCL by Olympus, Tokyo, Japan), which have a nominal spring constant of 42 N/nm, resonant frequency of 300 kHz, and nominal tip radius of 7 nm. Image processing was done using the Nanoscope Imaging software (Veeco Instruments, Santa Barbara CA) and the NIH ImageJ software (available at http://rsb.info.nih.gov/ij, National Institutes of Health, Bethesda, MD). We report the height and width of the GAG region of adsorbed aggrecan molecules for the different salt solutions. We note that the height reported in this article is the maximum height along a selected cross section of the molecule, subtracted from the average height of the substrate in the neighborhood. Figure 2 illustrates this procedure. For sampling consistency, all height and width measurements were performed along two cross sections of each aggrecan bottlebrush, and was limited to molecules having core length $>$ 100 nm in a 3 μm $\times$ 3 μm imaging area. We report the average and standard deviation of the measurements made on 40 to 80 adsorbed aggrecan molecules, for each salt condition. Statistical significance was measured using the student *t*-test.

RESULTS

Adsorption of Aggrecan on APS-Mica and Untreated Mica

Figure 3 shows aggrecan adsorbed onto APS-mica and onto untreated mica from the three different salt solutions. On the positively charged APS-mica [Fig. 3(a–c)], aggrecan adsorbs under all salt conditions, though the extent of adsorption is smaller from pure (salt-free) water, than from the salt solutions. The average number of aggrecan molecules adsorbed per μm^2 from the three salt conditions are 4.5 (pure water), 9.6 (0.1 *M* NaCl) and 11.1 (0.1 *M* NaCl + 0.002 *M* $CaCl_2$), respectively. There is a broad size distribution of the adsorbed molecules. For all salt conditions, aggrecan spreads out with visible GAG chains. The structure of the adsorbed aggrecan is qualitatively similar to that reported for positively-charged APTES mica in 0.1 M NaCl.[7] Distinct globular domains are clearly visible as bright spots along the aggrecan protein core (diameter ~15–25 nm, height ~0.9–1.5 nm). (We note that the surface of the APS-mica is hydrophobic as indicated by its wetting properties.)

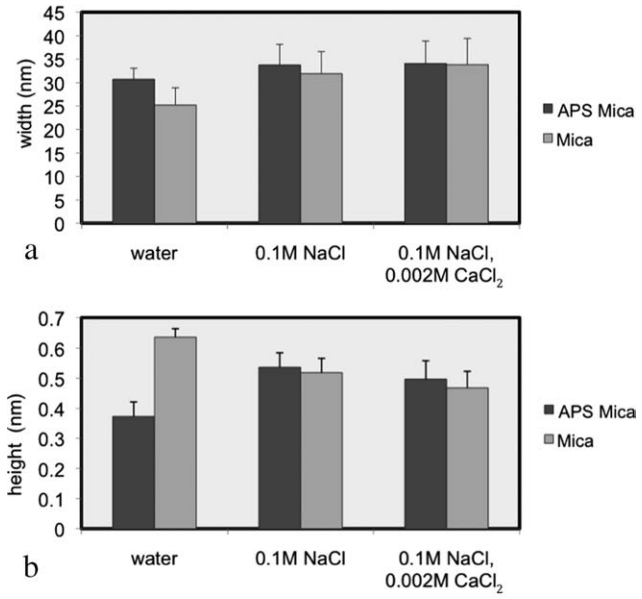

FIGURE 4 Width (a) and height (b) of aggrecan molecules adsorbed on untreated mica and APS-mica from different salt solutions.

On the negatively charged mica surface, however, the aggrecan adsorption varies for the different salt conditions [Fig. 3(d–f)]. As mentioned earlier, aggrecan did not adsorb onto the plain mica surface from the salt-free solution. Therefore, the aggrecan droplet was air dried on the surface and imaged without prior rinsing. The aggrecan molecules dried on the surface do not show distinguishable GAG chains [Fig. 3(d)]. Addition of 0.1 *M* NaCl makes the GAG chains distinguishable [see Fig. 3(e)], and this finding is comparable to that observed on APS mica despite the difference in charge and hydrophobicity between the two surfaces. However, when 0.002 *M* $CaCl_2$ is added, the GAG chains are no longer distinguishable [Fig. 3(f)]. The globular domains remain visible for all salt conditions. The adsorption of negatively charged aggrecan onto negatively charged mica can be attributed to specific interactions between the charged polymer chains and the surface. In addition, divalent counterions may neutralize the charge of both the aggrecan and the mica. However, the detailed mechanism of the ion-exchange and adsorption processes is not fully understood.

Figure 4(a,b) show the average width and height of aggrecan molecules adsorbed on untreated and APS-mica surfaces under different salt conditions. Assuming that the adsorbed polymers have similar intrinsic variation in GAG chain length, the average width indicates how extended the GAG chains are on each surface. Furthermore, assuming similar GAG and core-protein widths for the adsorbed molecules, the imaged height reflects the degree of GAG extension (aggrecan molecules with well-spread and adsorbed GAG chains appear lower than those with unextended GAG chains). On APS mica, no significant difference can be detected between the average widths of the aggrecan molecules adsorbed from

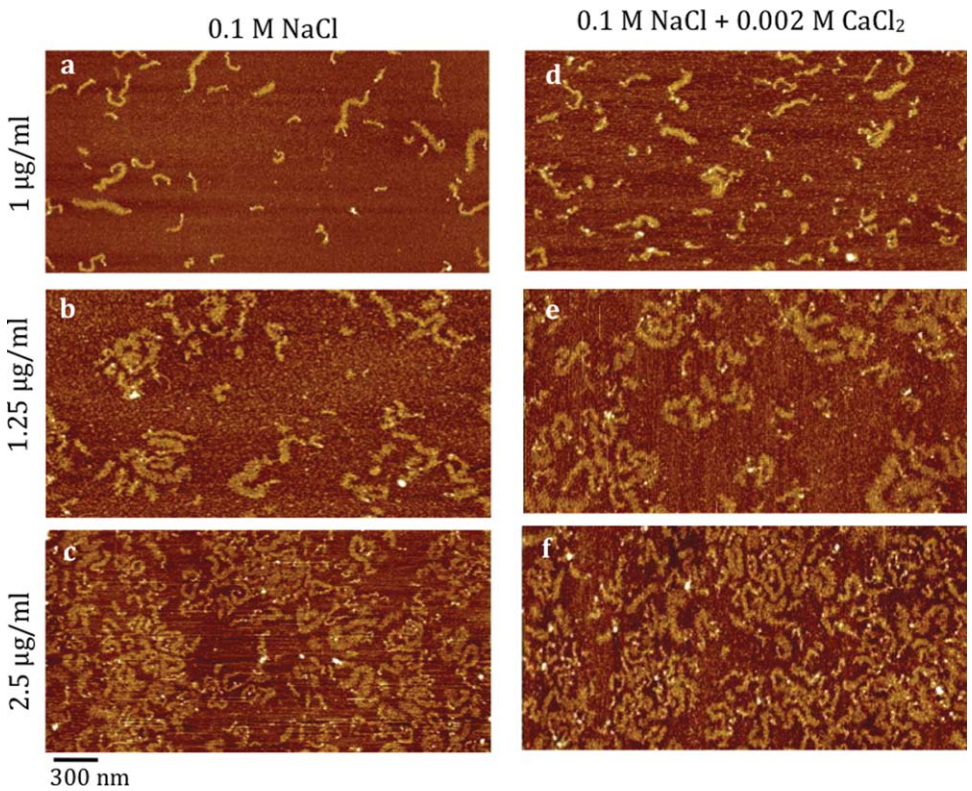

FIGURE 5 Aggrecan adsorbed on APS-coated mica shows distinct adsorption patterns at different concentration regimes. At dilute concentrations (~1 μg/mL), the aggrecan molecules are well dispersed. At higher concentration (1.25 μg/mL) aggrecan forms clusters, which coexist with dispersed entities. At even higher concentrations (>2.5 μg/mL), the clusters become larger, and eventually form a monolayer completely covering the substrate. Scan area: 3 × 1.5 μm^2; scan frequency: 1.5 Hz. scale bar: 300 nm.

different salt solutions [Fig. 4(a)]. However, the height of the molecules on the APS mica surface adsorbed from salt-free solution appears significantly smaller than those adsorbed from salt solutions ($p < 0.0001$) [Fig. 4(b)]. In the case of the mica surface, there is no appreciable difference between the average height and width of aggrecan molecules adsorbed from 0.1 M NaCl with or without $CaCl_2$. The dimensions of the adsorbed molecules in the presence of salt are similar to those measured on APS-mica surface. However aggrecan molecules dried on the mica surface from pure water exhibit significantly smaller width ($p < 0.0001$) and larger height ($p < 0.0001$) than molecules adsorbed from salt solutions, suggesting that the GAG chains are condensed on the protein core.

Adsorption Patterns with Incremental Aggrecan Concentration

As aggrecan molecules adsorb strongly onto APS mica, we study the effect of concentration on the adsorption patterns on this substrate. Figure 5(a–c) show adsorbed aggrecan molecules from 0.1 *M* NaCl solutions and for aggrecan concentrations of 1.0, 1.25, and 2.5 μg/mL, respectively. At 1.0 μg/mL, the aggrecan molecules are well dispersed and adsorb as individual entities. At 1.25 μg/mL clusters and

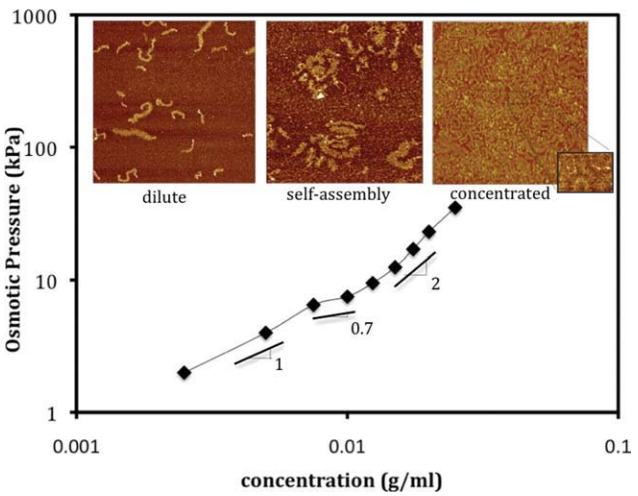

FIGURE 6 Comparison between the changes in aggrecan adsorption patterns with increasing surface concentration and the concentration dependence of the osmotic pressure of aggrecan solutions.[11] The surface images for the dilute, self-assembly, and concentrated states correspond to 1.0, 2.5, and 10.0 μg/mL, respectively, of adsorbing aggrecan in 0.1 *M* NaCl solution. Scan area: 1.5 × 1.5 μm^2; scan frequency: 1.5 Hz.

dispersed entities coexist. At higher concentration ($c \geq 2.5$ μg/mL) aggrecan forms extended clusters and the entire surface is eventually covered by a monolayer of conforming polymers. It is notable that aggrecan in the presence of salts interacts strongly with the substrates but any interaction among aggrecan molecules is much weaker as indicated by the absence of aggrecan overlap. Figure 5(d–f) show that the adsorption patterns from aggrecan solutions containing 0.002 *M* $CaCl_2$ are similar to those observed in the absence of calcium ions.

CONCLUSIONS

The morphology of aggrecan molecules adsorbed on charged surfaces, and the change in the adsorption pattern with increasing concentration has been investigated. The quantitative features of adsorbed molecules, such as the average height and width of their GAG regions are compared under three different salt conditions: (a) pure water, (b) 0.1 *M* NaCl, (c) 0.1 *M* NaCl containing 0.002 *M* $CaCl_2$.

On APS-mica in the absence of salt, aggrecan adsorbs with extended GAG chains indicating that the adsorption process is governed by attractive electrostatic interactions between the negatively charged GAGs and the positively charged surface. Adsorbed aggrecan molecules show at least four characteristic features. (1) The GAG side chains of molecules adsorbed from 0.1 M NaCl solution are distinguishable and extended on the surface. (2) The average height and width of the GAG chains do not change significantly in the presence of calcium ions. (3) Aggrecan adsorbed from 0.1 *M* NaCl displays distinct adsorption patterns with increasing concentration. Addition of $CaCl_2$ does not affect the adsorption patterns. (4) With increasing surface concentration, aggrecan adsorption patterns gradually change from that of separate molecules to clusters of bottlebrushes, and then to a monolayer of densely packed conforming chains.

The variation of the adsorption pattern shows similarities with concentration dependent changes of solution osmotic properties. At low concentration, the osmotic pressure of the aggrecan solution resembles that of dilute polymers, that is, the Π increases linearly with the aggrecan concentration. Correspondingly, aggrecan from dilute solutions ($c < 1$ μg/mL) adsorbs on the mica surface as individual entities [see Fig. 5(a)]. At higher concentration the osmotic pressure exhibits a weaker than linear concentration dependence indicating self-assembly among the aggrecan bottlebrushes. Correspondingly, aggrecan adsorbed in the intermediate concentration range ($2.5 < c < 5$ μg/mL) forms clusters as shown in Figure 5(b,c). The cluster formation can be attributed to the difference in water affinity between the globular domains of the protein core of the aggrecan molecule and the highly charged, hydrophilic polysaccharide bristles. In the semidilute concentration regime, Π is no longer due to the number of individual molecules but to the repulsion between each molecule and its nearest neighbor, and is thus approximately proportional to the square of the aggrecan concentration, that is, $\Pi \propto c^2$. Correspondingly, at aggrecan concentrations > 5 μg/mL the adsorbed clusters extend and form a continuous monolayer of conforming chains (Fig. 6). The continuous line in Figure 6 illustrates the osmotic profile of aggrecan solutions with distinct change in the power-law behavior in the different concentration regimes.[11] For illustrative purposes, we show alongside the '*surface equivalent*' of the aggrecan solutions (1.0, 2.5, and 10.0 μg/mL, respectively).

Aggrecan adsorption exhibits remarkable differences on untreated mica and APS-mica surfaces. Aggrecan from pure water do not absorb on untreated mica. When air-dried onto the surface, the GAG chains are indistinguishable. The average width of the bottlebrush is smaller, and its average height is larger than that of molecules adsorbed with extended GAG chains. In the case of adsorption from 0.1 M NaCl, however, the GAG chains become visible. In the presence of $CaCl_2$ the average height and width of the molecules are similar to those on APS mica, but the GAG chains are not distinguishable by AFM.

The insensitivity of the morphology of adsorbed aggrecan assemblies to the ionic environment and the surface properties of the substrate allows aggrecan to act as an ion reservoir playing a critical role in cartilage/skeletal metabolism.

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